Medical Staff Conference

The Design of Rational Combination Chemotherapy for Cancer

These discussions are selected from the weekly staff conferences in the Department of Medicine, University of California, San Francisco. Taken from transcriptions, they are prepared by Drs Homer A. Boushey, Associate Professor of Medicine, and David G. Warnock, Associate Professor of Medicine, under the direction of Dr Lloyd H. Smith, Jr, Professor of Medicine and Chairman of the Department of Medicine. Requests for reprints should be sent to the Department of Medicine, University of California, San Francisco, School of Medicine, San Francisco, CA 94143.

DR SMITH:* *Cancer is an extraordinarily important group of diseases not only because of the statistics of mortality, but because of the special dread that it often elicits. Peyton Rous, who won a Nobel prize for his pioneering work concerning viruses and cancer, wrote as follows: "Tumors destroy man in an unique and appalling way, as flesh of his own flesh, which had somehow been rendered proliferative, rampant, predatory and ungovernable." Fortunately there has been remarkable progress in the treatment of various cancers over the past generation. Much of this has been based on empirical studies, but increasingly these therapeutic protocols are being based on considerations that will be described in this Medical Staff Conference.*

Our speaker will be Dr Edwin C. Cadman, Professor of Medicine and Director of the Cancer Research Institute. Dr Cadman joined our faculty in the above capacity only a few months ago following a distinguished career in oncology at Yale.

General

DR CADMAN:† Cancer is the second leading cause of death in this country, exceeded only by deaths due to heart disease. About 440,000 people will die of cancer this year, and there will also be 1.2 million new cases of cancer diagnosed.[1] Although these numbers appear somewhat discouraging, it is estimated that this year about 50,000 patients will be cured of their cancer by the use of chemotherapy.[2] Cure is defined as achieving a state wherein chemotherapy can be discontinued after a maximum of six months of treatment and the patient can return to a normal and productive life expectancy without further need for treatment. The only other diseases that an internist can cure with medication as just defined are the infectious diseases. The unfortunate nature of these curable cancers, however, is that they comprise less than 10% of the total number of cancers diagnosed in this country (Table 1). The one major difference that separates these curable cancers from the noncurable cancers, such as lung cancer, colon cancer, cancer of the pancreas and the other more common neoplastic diseases, is that the curable cancers tend to have doubling times of about 30 to 60 days.

Most of the drugs that are available for current use by oncologists were developed 15 or 20 years ago, some as many as 25 to 30 years ago. These drugs were designed primarily to interfere with DNA synthesis because at the time of their development normal DNA structure and function were considered the most crucial requirement for cell division and therefore tumor growth. This knowledge helps explain why most of the current antineoplastic agents are most effective in rapidly proliferating tumors. In fact, all of these drugs were screened against and were required to kill rapidly proliferating mouse leukemia and lymphoma cells that had doubling times of 12 hours. Current drug development concepts involve the creation of agents that may have selective activity against cancer cells compared with normal cells. This novel approach to the design of newer antineoplastic drugs is permissible because malignant cells often contain unique cellular proteins and occasionally different quantities of cell surface antigens. Even the older drugs, if attached to monoclonal antibodies specific for different antigenic substances on malignant or transformed cells, could theoretically de-

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ABBREVIATIONS USED IN TEXT

dTMP = deoxythymidylate
dTTP = deoxythymidine triphosphate
dUMP = deoxyuridylate
FdUMP = fluorodeoxyuridylate
FdUTP = fluorodeoxyuridine triphosphate
FH_2 = dihydrofolate
FH_4 = tetrahydrofolate
5-FU = 5-fluorouracil
FUMP = 5-fluorouridylate
FUTP = 5-fluorouridine triphosphate
HGPRTase = hypoxanthine-guanine phosphoribosyltransferase
OPRTase = orotate phosphoribosyltransferase
PRPP = 5-phosphoribosyl-1-pyrophosphate
TMP = thymidylate (thymidine monophosphate)
TTP = thymidine triphosphate
UMP = uridylate (uridine monophosphate)
UTP = uridine triphosphate

liver the toxic agent selectively to the desired site. These are purely concepts, however, that remain to be developed and evaluated. The issue an oncologist must consider is how to optimally treat a patient whose cancer is diagnosed today.

Chemotherapy for malignant disease is the result of medical knowledge that was accumulating in the 1940s. Infectious diseases were finally being cured with antibiotics. This provided a major impetus for the development of drugs to cure cancer. In addition, the DNA structure was being identified, and the precise nature of its activity was found to be crucial to cellular proliferation. Therefore drugs that interfered with this nucleic acid were thought to have particular interest as anticancer drugs. What was not appreciated then was that cancer cells were more like normal cells than they were different, and this selective destruction of malignant cells was not often achieved. The excitement that followed antibiotic treatment of bacterial disease was replaced by disappointment when toxic effects on normal tissue became dose limiting after drug treatment for cancer was introduced.

Mechlorethamine hydrochloride, a nitrogen mustard, was the first drug used, in 1943, to treat patients who had Hodgkin's disease, after it had been documented that lymph nodes disappeared in soldiers exposed to mustard gas.[3] The result was a dramatic size reduction of cancerous lymph nodes, the duration of which was only temporary. In 1947 it was established that administration of the antifolate, methotrexate, could result in rapid elimination of leukemic lymphocytes in children with acute lymphocytic leukemia.[4] In 1955 this same drug was associated with the first cures in women who had gestational choriocarcinoma.[5] With the aforementioned theoretical reasoning and with these successes, chemotherapy for malignant disease seemed a very worthwhile endeavor.

Drug development has been a laborious and time-consuming process. This effort is sponsored primarily by the National Cancer Institute, though recently the pharmaceutical industry has been investing in drug

TABLE 1.—*The Curable Cancers**

Cancer in Adults	*% Cured*	*Cancer in Children*	*% Cured*
Hodgkin's disease	80	Acute lymphocytic leukemia	60
Diffuse histiocytic lymphoma	50	Embryonal rhabdomyosarcoma	80
Nodular mixed lymphoma	50	Burkitt's lymphoma	50
Testicular carcinoma	80	Wilms' tumor	80
Gestational choriocarcinoma	80	Ewing's sarcoma	70
Ovarian carcinoma	10		
Acute myelogenous leukemia	10		

*From "Cancer Statistics 1984"[1] and Frei.[2]

research processes. Over the past 20 years, 700,000 agents have been screened for possible chemotherapeutic use under the direction and funding of the National Cancer Institute.[2] These include 350,000 compounds that were synthesized, some for the specific purpose of interrupting cellular metabolism. About 200,000 were obtained from fermentation products and about 120,000 extracts of plant products were tested. The remaining compounds were simply miscellaneous agents. The process of drug acquisition requires a uniform screening procedure before the drugs can become available to treat patients. Drugs must be evaluated in test tube systems against several cultured malignant cell lines. If a drug shows some inhibitory activity against these tumor cells, it is then tested in small rodents containing tumors. It these tumors are successfully treated with the agent, then the drug is produced in sufficient quantities to allow toxicology testing to be done in large animals. Once the toxicology is understood, the drug is eventually allowed to be used in clinical trials.

The clinical testing program has the following three phases: Phase I is the use of these agents in patients who have a malignant tumor and in whom either standard therapy has failed or for whom no therapy exists. The disease is required to be far advanced and a patient's life expectancy to be less than two months. This study is specifically designed to learn the effects of these drugs—primarily their toxicity—on humans. The goal is to determine the safe drug dose for humans. During this particular phase, pharmacokinetic evaluations are often done and the dose and timing of drug administration estimated. Because of the escalating nature of dose administration in these clinical studies, only about 15 to 20 patients are required to establish a reasonably safe drug dose. The far-advanced nature of the cancer in these patients precludes evaluating these agents for therapeutic efficacy during this phase of clinical testing.

Phase II clinical evaluation is to identify the potential therapeutic usefulness of the new agent. Similar patients are selected for these studies as were for the phase I trials, with the exception that their life expectancy should be about six months or longer. Patients are administered drug doses that were selected in the phase I trial to be safe. Repeated treatments are given, with the primary objective being the documentation of

TABLE 2.—*Chemotherapeutic Drug Classification*

Alkylating Agents	
Cyclophosphamide	Lomustine (CCNU)
Melphalan	Semustine (methyl CCNU)
Busulfan	Streptozocin
Carmustine (BCNU)	Cisplatin
Antimetabolites	
Methotrexate	5-Azacitidine
5-Fluorouracil	Cytarabine
6-Mercaptopurine	Hydroxyurea
6-Thioguanine	
Antibiotics	
Doxorubicin hydrochloride	Bleomycin sulfate
Daunorubicin hydrochloride	Dactinomycin
Mithramycin	
Plant Alkaloids	
Vincristine sulfate	Etoposide (VP-16-123)
Vinblastine sulfate	
Miscellaneous	
Procarbazine hydrochloride	Hexamethylmelamine
Dacarbazine (DTIC)	L-Asparaginase

tumor responses. Drugs should be tested in all tumor types, but this is impractical. Drugs are often tested in only about five to ten common tumor types—lung cancer, ovarian carcinoma and breast cancer. In the phase III clinical trial, the new drug therapy is compared with the standard therapy for that particular cancer that responded to the new drug in the phase II testing. This last phase of clinical testing is often a randomized study.

This entire acquisition, screening and clinical testing program can take, on the average, about ten years. Therefore, drugs that are currently being developed will not be available for routine clinical use until the mid-1990s. These clinical cancer trials require the commitment of a compassionate physician, for often the best that can be offered to those willing patients is empathy and hope.

The common available chemotherapeutic antineoplastic agents are listed in Table 2. There are about 50 agents available for use in the treatment of cancer, including experimental drugs and hormones. It is well established that combination drug therapy for cancer almost always results in a higher response rate. In fact, all of the cancers listed in Table 1 are curable with combination therapy (to the percentages indicated only). Therefore, it is reasonable and appropriate to believe that combination therapy is better for achieving tumor responses, long-term remissions and cures in cancer patients. The problem is, of course, deciding the best and most logical method by which to select a drug combination. The following are often considered during this drug selection process: tumor cell kinetics and cell cycle activity of the drugs (use drugs that interfere with DNA synthesis if the tumor is growing quickly), mechanisms of drug resistance (do not use drugs that act at the same enzyme site or use the same carrier protein to enter the cell) and host toxic reactions (do not use drugs with the same toxicity). The major choice of drugs for use in combination therapy is, unfortunately, arbitrary or random. This capricious approach to the design of cancer therapy is in need of gentle direction. If there are 50 drugs from which to select a three-drug combination, nearly 20,000 different drug combinations can be randomly chosen. This large figure does not include varying the drug dose or frequency of drug administration. A random, unscientific approach to the design of drug combination cancer treatment could overwhelm the capacity of our academic centers, resulting in chaos and eventually in disappointment.

Biochemical Modulation

A more scientific approach to selecting drug combinations should be attempted to avoid the confusion and dismay that result from random choice. Biochemical modulation is a rational method of selecting drugs for combination trials. The effect of one drug on the intracellular metabolism and the consequence of these perturbations on a second drug constitute the foundation of the principles of biochemical modulation. Our research laboratory has been investigating the interaction of methotrexate and 5-fluorouracil (5-FU) to determine biochemically the most rational way to combine these two agents. These drugs were chosen for evaluation for two reasons. The first was that they were antimetabolites that affected nucleotide metabolism and therefore would be capable of being modulated within cells by alterations in intracellular nucleotide levels. The second was that these drugs are perhaps the two most common drugs used for treatment of the most common and incurable cancers: cancer of the colon and the breast. Our studies have shown that when methotrexate is given before 5-FU, enhanced intracellular accumulation of 5-fluorouracil occurs, which is associated with synergistic killing of cancer cells. The following is an explanation of this drug interaction that shows how basic laboratory information can be of use in the design of clinical treatment.

Interaction of Methotrexate and 5-Fluorouracil

Effect of Methotrexate

Methotrexate is an antifolate drug that inhibits the enzyme dihydrofolate reductase. The consequence of this inhibition is the prevention of the regeneration of the biologically active tetrahydrofolate (FH_4) from the biologically inactive dihydrofolate (FH_2). Deoxythymidylate (dTMP), the precursor of deoxythymidine triphosphate (dTTP), one of the four nucleoside triphosphates required for DNA synthesis, is created simply by the transfer of the methyl group from the folate, 5,10-methylenetetrahydrofolate, to deoxyuridylate (dUMP). This methyl group to be donated to dUMP is carried by the tetrahydrofolate structure, which loses two hydrogens during the transfer process, leaving the biologically inactive oxidized dihydrofolate (Figure 1). Therefore, when dihydrofolate reductase is inhibited by methotrexate, the synthesis of dTMP from dUMP consumes the FH_4 by converting it to FH_2, which can no longer be converted back to the active

FH_4.[6] When the FH_4 levels are reduced below concentrations that will support the synthesis of dTMP, then DNA synthesis ceases. Obviously, methotrexate would only be effective in cells that required dTTP, or are synthesizing DNA.

Intracellular folates are also necessary for one-carbon metabolism in the de novo purine synthetic pathway. The formation of formylglycinamide ribonucleotide from glycinamide ribonucleotide requires 5,10-methenyl FH_4. The synthesis of 5-formaminoimidazole-4-carboxamide ribonucleotide requires the transfer of the formyl group from 10-formyl tetrahydrofolate to 5-aminoimidazole-4-carboxamide ribonucleotide. Neither one of these folate-requiring steps results in the oxidation of the FH_4 structure to the biologically inactive FH_2 derivative. Therefore, the FH_4 compound that results after the one-carbon transfer during purine synthesis can obtain other one-carbon units required for further purine synthesis without the requirement of the enzyme dihydrofolate reductase. The presumed effect of methotrexate on the purine synthetic pathway is thus an indirect one and the consequence of the altered folate pools that result from continued dTMP synthesis in the presence of dihydrofolate reductase inhibition. When dihydrofolate reductase is inhibited by methotrexate, $K_i \simeq 10^{-11}$ M[7]; 5,10-methylene FH_4 is utilized by the conversion of dUMP to dTMP, producing FH_2 until the folate cofactor, 5,10-methylene FH_4, can no longer support this reaction at a rate that sustains DNA synthesis. Presumably a sufficient amount of FH_4, which would have undergone conversion to 5,10-methenyl FH_4 or 10-formyl FH_4 for purine synthesis, is now transformed to 5,10-methylene FH_4 in an attempt to continue the synthesis of the needed dTMP. The response to the inhibition of dihydrofolate reductase by methotrexate therefore results in a reduction in de novo purine synthesis because of this continued use without replenishment of FH_4 (Figure 2). The influence of methotrexate on the synthesis of purines had been appreciated in 1958[8]; however, the cytotoxic effect of methotrexate was always considered the result of a thymineless state.[9,10]

The following experimental evidence substantiates the validity of this effect of methotrexate on purine synthesis. Glycine is normally incorporated into the purine ring during formation of the purine structure glycinamide ribonucleotide from phosphoribosylamine. Glycine, which has a ^{14}C label at the 1 position of the molecule, can be used to measure the rate of purine synthesis. In unperturbed cell growth the 1-^{14}C label can be found in the purine nucleotides that are incorporated into DNA and RNA. However, in the presence of methotrexate at concentrations that inhibit dihydrofolate reductase maximally for at least one cell doubling time, the ^{14}C label was substantially reduced in these purine nucleotides, indicating a reduction in the new synthesis of adenine and guanine, the purines. This observation is consistent with the proposed indirect inhibition of de novo purine synthesis by methotrexate (Figure 3).[11]

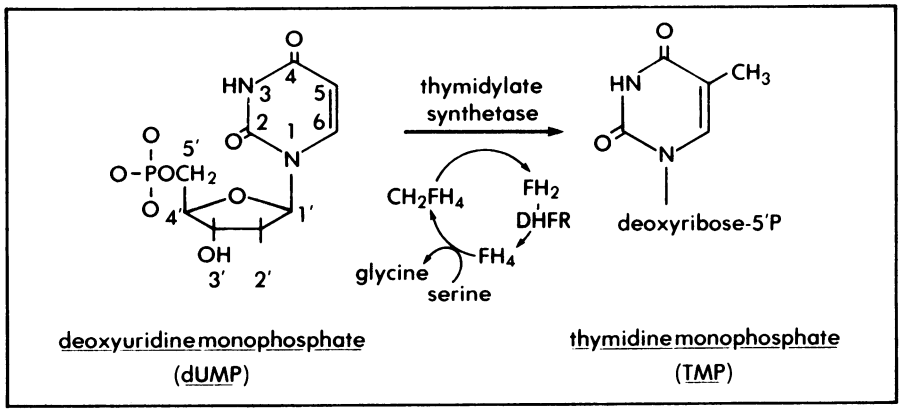

Figure 1.—The conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP). Deoxyuridylate, or dUMP, obtains a methyl group in the 5 position of the base ring from the folate, 5,10-methylenetetrahydrofolate (CH_2FH_4). The result is TMP and the oxidized folate, FH_2. This latter folate is inactive and must be converted to tetrahydrofolate (FH_4) to sustain further TMP synthesis. DHFR = dihydrofolate reductase

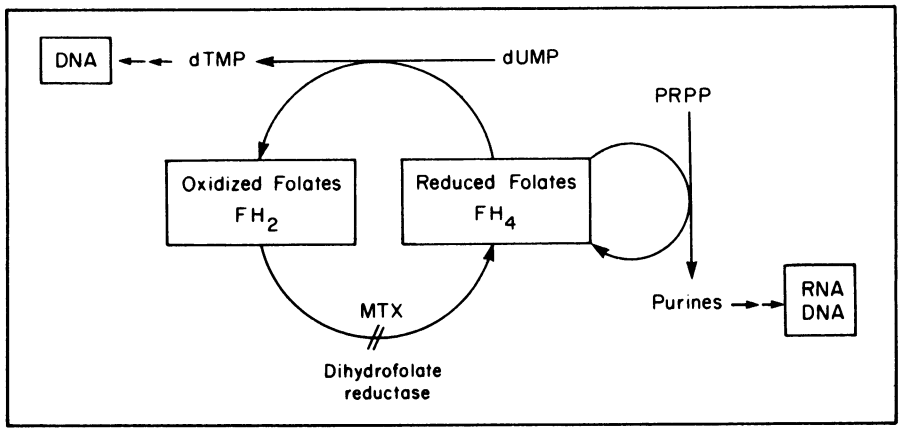

Figure 2.—The mechanism by which methotrexate (MTX) affects intracellular folate levels. Normally the reduced folates (FH_4) carry one carbon group to the de novo purine synthetic pathway and return unaltered to the FH_4 pool. When the FH_4 moiety transfers a carbon group to deoxyuridine monophosphate (dUMP), forming thymidylate (dTMP), the FH_4 loses two hydrogens (is oxidized). This biologically inactive folate (FH_2) must obtain two hydrogens (be reduced) by dihydrofolate reductase (DHFR). PRPP = phosphoribosylpyrophosphate

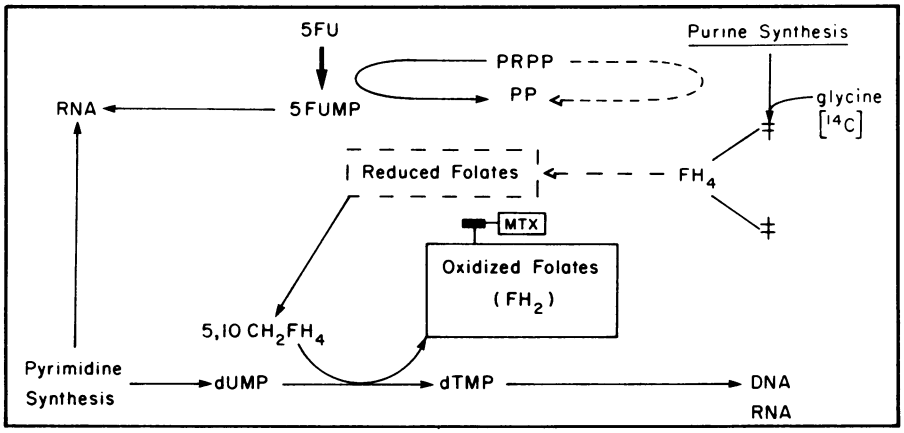

Figure 3.—The mechanism by which methotrexate (MTX) results in enhanced intracellular accumulation of 5-fluorouracil (5-FU). When methotrexate inhibits dihydrofolate (FH_2) reductase, FH_2 cannot be converted back to the biologically active tetrahydrofolate (FH_4). Consequently, purine synthesis ceases, phosphoribosylpyrophospate (PRPP) increases, which in turn can be used for the metabolic conversion of the base, 5-FU, to the nucleotide derivative, 5-FUMP. PP = pyrophosphate, 5,10-CH_2FH_4 = 5,10-methylenetetrahydrofolate, dTMP = thymidylate, dUMP = deoxyuridylate

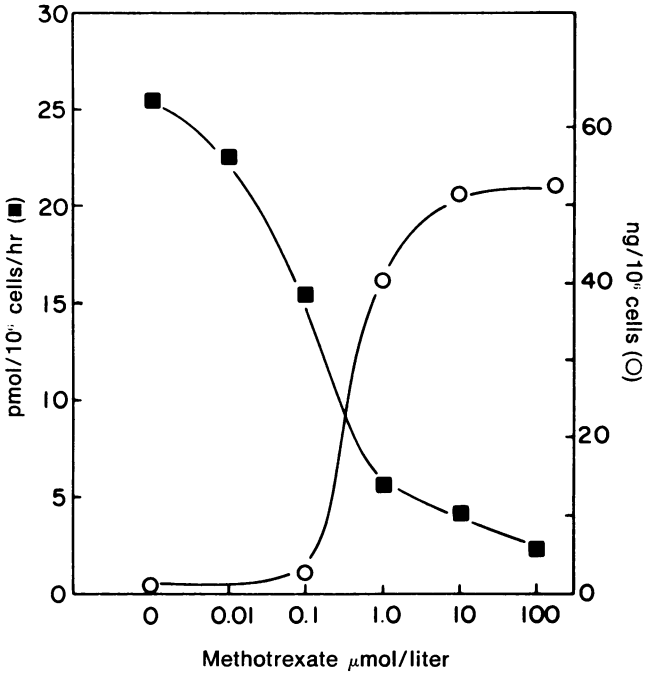

Figure 4.—The effect of increasing methotrexate concentrations for a six-hour exposure on the synthesis of purine bases and phosphoribosylpyrophosphate (PRPP) levels in L1210 mouse leukemia cells. Single cell suspensions of L1210 cells were exposed to the indicated concentration of methotrexate. After six hours the amount of radiolabeled glycine that accumulated into adenine and guanine over two hours was determined. Simultaneous determinations of PRPP were also done. ○ = PRPP, ■ = 1-^{14}C-glycine into adenine and guanine

The consequence of this inhibition on the de novo purine pathway is an increase in the availability of 5-phosphoribosyl-1-pyrophosphate (PRPP), which normally contributes the phosphoribosyl moiety with the amino group donated by glutamine to form phosphoribosylamine. This is the first enzymatic process in the synthesis of purines. The PRPP can also be used by orotate phosphoribosyltransferase (OPRTase), an enzyme used in the de novo pyrimidine synthetic pathway, as well as by hypoxanthine-guanine phosphoribosyltransferase (HGPRTase) and adenine phosphoribosyltransferase (APRTase), enzymes used in the salvage of purines. In each enzymatic conversion, the phosphoribosyl moiety of PRPP is combined with the respective base. For example, HGPRTase can only utilize hypoxanthine or guanine. The activity of these enzymes can be influenced by the availability of their obligatory cosubstrate, PRPP, and the base to be converted to a nucleotide. 5-Fluorouracil is a base analog of uracil and requires PRPP for activation or conversion to the nucleotide fluorouridylate (FUMP). Therefore 5-FU activation can be influenced by an increase in PRPP levels, which can be achieved following methotrexate treatment. In Figure 4 is shown that in L1210 mouse leukemia cells, as the concentration of methotrexate is increased over an exposure of six hours, there is a resultant decrease in the amount of radiolabeled glycine in the purines. This reduction in purine synthesis in response to methotrexate is accompanied by a concomitant increase in the intracellular concentrations of PRPP.

The Enhancement of 5-Fluorouracil Accumulation in Cancer Cells by Methotrexate

5-Fluorouracil is identical to uracil, except that on the 5 position of the 6-membered pyrimidine ring there is a fluorine atom; this is precisely where the methyl group was to have been placed during dTMP synthesis (Figure 1). 5-Fluorouracil enters the cells by facilitated diffusion and then is converted to the monophosphate nucleoside, FUMP (Figure 3). The mechanism by which 5-FU kills cells will be discussed later.

The conversion of 5-FU to 5-fluorouridylate (FUMP) is by OPRTase. This initial ribosylphosphorylation is considered to be rate limiting in the total intracellular accumulation of 5-FU ribonucleotide derivatives and therefore the important enzymatic process that could be modulated. This enzyme, which activates 5-FU, requires PRPP from which the phosphoribosyl moiety is transferred to 5-FU to form FUMP. It is from this monophosphate derivative of 5-FU that the subsequent formation of other 5-FU nucleotides occurs.

After methotrexate exposure and the elevation of PRPP levels, 5-fluorouracil can be converted in greater quantity to FUMP by OPRTase. When methotrexate was given to L1210 cell cultures at concentrations that saturated dihydrofolate reductase, generally greater

than 1 μmol per liter, deoxythymidine triphosphate (dTTP) pools were reduced, purine synthesis was inhibited and PRPP levels increased fivefold to tenfold (7 ng per million cells to 52 ng per million cells after a three-hour exposure to methotrexate). When 5-FU was added to these methotrexate-exposed cells, the rate and total intracellular accumulation of 5-FU metabolites increased fivefold.[11-13] The biological significance of this enhanced 5-FU accumulation in methotrexate-pretreated cells was synergistic cell killing.

Important factors that can influence this biochemical modulation between methotrexate and 5-FU are the dose of methotrexate, the interval between the administration of methotrexate and that of 5-FU, the population of tumor cells undergoing DNA synthesis and the presence of other compounds that could use and therefore consume PRPP. For example, FH_4 depletion will occur only in cells exposed to methotrexate that are synthesizing dTMP and therefore utilizing 5, 10-methylene FH_4. Hypoxanthine is also a base that is converted to inosine monophosphate in the presence of PRPP by HGPRTase. The inosine monophosphate enters the purine pathway beyond where the folate carbon transfers are required for purine synthesis. Therefore hypoxanthine can both use PRPP and sustain the production of adenine and guanine in the presence of methotrexate. Consequently, cells exposed to high concentrations of hypoxanthine after methotrexate treatment will not accumulate large quantities of 5-FU nor will there be synergistic cytotoxicity. This has clinical implications because some patients have sufficient serum concentrations of hypoxanthine to blunt the biochemical modulatory effects described. Allopurinol, an analog of hypoxanthine that also requires PRPP for activation and, in addition, will increase hypoxanthine serum levels, has the potential to completely inhibit the conversion of 5-FU to its active derivatives. Finally, leucovorin, which is an exogenous source of FH_4, will rapidly reverse the effects of the methotrexate inhibition on dihydrofolate reductase. If leucovorin is administered after the methotrexate but before 5-FU, the intracellular biochemical alterations described and enhanced cytotoxicity of the methotrexate/5-FU sequence will also be totally abrogated.

The sequential administration of methotrexate before 5-FU was initially studied in L1210 cells. Similar modulation of 5-FU metabolism and enhanced cytotoxicity has also been observed in the human colon carcinoma cell line HCT-8 and the human breast cancer cell line 47-DN.[13,14] In Figure 5 the results of these studies in human breast cancer cells are summarized. Although the maximum effect on 5-FU metabolism—PRPP elevation—and cytotoxicity of sequential methotrexate and 5-FU administration occurred between three and six hours for the L1210 cells, the optimal methotrexate exposure duration to potentiate 5-FU accumulation for the human breast cancer cell line occurred after 24 hours. Knowing this duration of methotrexate exposure for the maximum effect on 5-FU activation and cytotoxicity is important when this information is being used to design sequential therapy for cancer patients. The most obvious reason for this difference required for optimal biochemical modulation is that the L1210 cells have a 12-hour doubling time, whereas the human cells have a doubling time of 24 to 36 hours. As previously noted, methotrexate is maximally effective in cells that are actively synthesizing DNA (making dTMP). Therefore, the longer the exposure to methotrexate of a slowly dividing cell population, the greater is the proportion of the total cell population that will begin to synthesize DNA and be affected by methotrexate. Other factors such as changes in methotrexate transport, polyglutamate formation of methotrexate and methotrexate retention may be related to growth patterns and may be

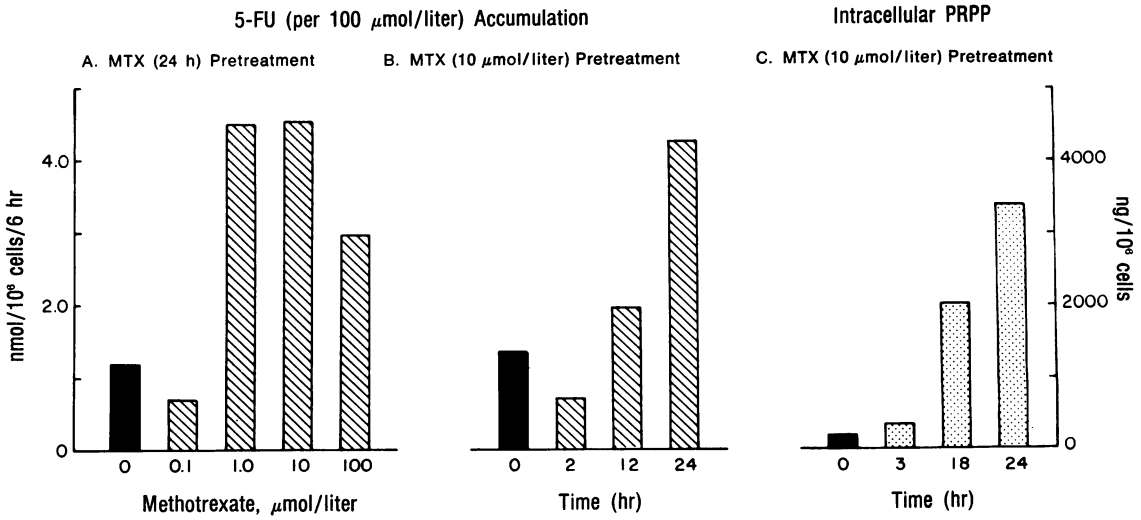

Figure 5.—The effect of methotrexate (MTX) concentration and duration of exposure to achieve maximum intracellular accumulation of 5-fluorouracil (5-FU) and elevation of phosphoribosylpyrophosphate (PRPP) levels were determined in the human breast cancer cell line, 47-DN. The optimal MTX concentration **(A)** was between 1 and 10 microns. After 24 hours of MTX, the PRPP levels **(C)** were maximum (longer MTX exposures were not better), which correlated with the maximum intracellular 5-FU accumulation **(B)**. ■ = no treatment, ▨ and □ = pretreatment as indicated

quite important as well.[15,16] Mechanisms by which cells can be synchronized or encouraged to undergo division (enter S-phase) could possibly enhance the sequential use of methotrexate and 5-FU. Estrogen stimulation of the breast cancer cell lines will, in fact, enhance the cytotoxicity of this drug sequence. Although the doubling time is decreased by estrogen treatment in breast cancer cells that contain estrogen receptors, other factors could be occurring.[17,18] This information was used to design a phase I clinical study in which methotrexate was given by mouth every six hours for five doses to sustain documented serum methotrexate concentrations in the specified range of 1 to 10 μmol per liter. After the 24-hour methotrexate exposure, an intravenous injection of a standard 5-FU dose was given. There was no severe toxic effect.[19]

Our current ongoing clinical trial involves an infusion of methotrexate (540 mg per sq m of body surface for 36 hours), with an infusion of 5-FU (1.5 grams per sq m) begun at 24 hours and continued for 24 hours. These drug doses were chosen to achieve blood concentrations similar to those found to be optimum for biochemical alterations and cytotoxicity as noted by laboratory studies—1 to 10 μmol per liter of methotrexate and 1 μmol per liter of 5-FU. For 12 hours (between the 24th and 36th hours), the methotrexate and the 5-FU infusions overlap. Leucovorin is given to patients beginning at the 36th hour. The therapy regimen is repeated every two weeks. Currently, 16 patients have been treated in this fashion after giving their informed consent. There have been minimal toxic effects. Therefore we plan on using this drug sequence for early treatment of metastatic breast cancer.

We are unable to state with assurance that this sequential therapy is selective for destroying cancer cells. We have evaluated normal bone marrow stem cells obtained from patients having a bone marrow biopsy done for other reasons. The methotrexate/5-FU sequence does not result in an enhanced destruction of bone marrow stem cells that develop into granulocyte or macrophage colonies (D. Armstrong, PhD, and E. Cadman, MD, unpublished observations, 1984). This may reflect the fact that the bone marrow environment contains hypoxanthine levels five to ten times greater than those found in serum (S. Howell, MD, University of California San Diego, unpublished observations, 1984). Therefore, perhaps the bone marrow has selective protection.

The Mechanism of Increased Cell Killing

The precise mechanism by which these two drugs result in enhanced cell killing is unknown but could be the consequence of several factors. The primary nucleotide forms of 5-fluorouracil considered to be cytotoxic are the analog of dUMP, fluorodeoxyuridylate (FdUMP), and the analog of uridine triphosphate (UTP), fluorouridine triphosphate (FUTP).

Inhibition of thymidylate synthesis. The conversion of dUMP to dTMP occurs by the enzymatic process of methyl transfer mediated by the enzyme, thymidylate synthetase. The fluoropyrimidine analog, FdUMP, binds tightly to this enzyme. This 5-FU derivative cannot receive a methyl group because at the 5 position of the ring where the methyl group is to be transferred, a fluorine atom exists. Because this nucleotide form of 5-FU is increased in cells pretreated with methotrexate, the mechanism of its cytotoxic action is important to understand. However, the binding of FdUMP to this enzyme is ordered and requires 5,10-methylene FH_4 for covalent bonding and enzyme inhibition to occur.[20] Therefore, in the presence of methotrexate, the 5,10-methylene FH_4 levels are depleted and the subsequent administration of 5-FU will not result in greater binding and inhibition of thymidylate synthetase by FdUMP. This is not the mechanism of enhanced cell killing.

Interaction with RNA function. The 5-FU ribonucleotide, fluorouridine triphosphate (FUTP), can be incorporated into newly synthesized RNA in place of UTP. The presence of FUTP in RNA does inhibit the processing of the larger nuclear RNA into appropriate smaller ribosomal RNA subunits. The higher molecular weight RNA normally undergoes cleavage at various sites, leaving RNA molecules of the correct size and structure that are then transported into the cytosol where they carry out their normal function. This processing occurs extensively to that nuclear RNA that subsequently becomes ribosomal RNA. When certain quantities (the exact amount is unknown) of FUTP are incorporated into the newly synthesized RNA strands, this processing is inhibited.[21,22] Methotrexate pretreatment does result in enhanced incorporation of FUTP into RNA; therefore, this may be a mechanism that could account for the enhanced cell killing following the methotrexate/5-FU sequence.

Interaction with DNA structure and function. The deoxynucleotide that is also made from 5-FU fluorodeoxyuridine triphosphate (FdUTP), can be incorporated into DNA in place of dTTP. The DNA polymerase can utilize FdUTP equally as well as it can dTTP. The amount of FdUTP incorporated into DNA depends on the ratio of FdUTP to dTTP. However, DNA does not contain uracil. The reason is that the enzyme, DNA-uracil glycosylase, acts quickly to remove any uracil. This in turn initiates the DNA excision repair process that ultimately replaces the site of the uridine nucleotide with dTTP.[23]

If large amounts of FdUTP are incorporated into DNA, the DNA repair process will function at such a rapid and efficient rate that the DNA will have many areas lacking bases (or have "holes") and therefore become nonfunctional. Under certain circumstances, this can lead to DNA fragmentation (E. Cadman, MD, unpublished observations, 1984). Some tumor cells may have enhanced DNA repair and therefore, in fact, be very susceptible to this injury. This mechanism could result in selective destruction of tumor cells com-

pared with normal nondividing cells under the right conditions.

Conclusion

Biochemical modulation as exemplified by the interaction of methotrexate and 5-fluorouracil can be used as a rational guide for the selection of drug combinations to be used in the treatment of cancer. In cells that are synthesizing DNA—that is, rapidly proliferating tumors—pretreatment with methotrexate results in enhanced intracellular accumulation of subsequently administered 5-FU. This enhanced intracellular 5-FU accumulation is the consequence of the antipurine effect of methotrexate and the resultant elevation of PRPP, which is the cosubstrate required for the metabolic conversion of the base, 5-FU, to the nucleotide, FUMP. The observed synergistic cell killing of the sequential administration of methotrexate and 5-FU is associated with this increased intracellular 5-FU nucleotide levels. Although the initial biochemical and cytotoxic studies were done in L1210 cells, they have been reduplicated in cultured human breast and colon cancer cells. This information does provide a rational basis from which to make therapeutic decisions when using methotrexate and 5-FU for cancer treatment.

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